Adaptor protein p66(Shc) mediates hypertension-associated, cyclic stretch-dependent, endothelial damage

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Adaptor Protein p66^{Shc} Mediates Hypertension-Associated, Cyclic Stretch–Dependent, Endothelial Damage

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Abstract—Increased cyclic stretch to the vessel wall, as observed in hypertension, leads to endothelial dysfunction through increased free radical production and reduced nitric oxide bioavailability. Genetic deletion of the adaptor protein p66^{Shc} protects mice against age-related and hyperglycemia-induced endothelial dysfunction, as well as atherosclerosis and stroke. Furthermore, p66^{Shc} mediates vascular dysfunction in hypertensive mice. However, the direct role of p66^{Shc} in mediating mechanical force–induced free radical production is unknown; thus, we studied the effect of cyclic stretch on p66^{Shc} activation in primary human aortic endothelial cells and aortic endothelial cells isolated from normotensive and hypertensive rats. Exposure of human aortic endothelial cells to cyclic stretch led to a stretch- and time-dependent p66^{Shc} phosphorylation at Ser36 downstream of integrin α5β1 and c-Jun N-terminal kinase. In parallel, nicotinamide adenine dinucleotide phosphate oxidase activation, as well as production of reactive oxygen species, increased, whereas nitric oxide bioavailability decreased. Silencing of p66^{Shc} blunted stretch-increased superoxide anion production and nicotinamide adenine dinucleotide phosphate oxidase activation and restored nitric oxide bioavailability. In line with the above, activation of p66^{Shc} increased in isolated aortic endothelial cells of spontaneously hypertensive rats compared with normotensive ones. Pathological stretch by activating integrin α5β1 and c-Jun N-terminal kinase phosphorylates p66^{Shc} at Ser36, augments reactive oxygen species production via nicotinamide adenine dinucleotide phosphate oxidase, and in turn reduces nitric oxide bioavailability. This novel molecular pathway may be relevant for endothelial dysfunction and vascular disease in hypertension. **(*Hypertension*. 2014;64:347-353.)** ● **Online Data Supplement**

Key Words: endothelium ■ hypertension ■ nitric oxide ■ reactive oxygen species ■ stress, mechanical

Hypertension is an established risk factor for the development of cerebrovascular and cardiovascular disease[1–3]; in patients with hypertension, each increase of 1 mm Hg in blood pressure corresponds to a 1% increase in stroke mortality.[4] Hypertension exerts its deleterious effects on the vessel wall mainly via mechanical forces such as shear stress and cyclic stretch.[5] Under physiological conditions, mechanical forces are crucial for the maintenance of vascular cell homeostasis[6]; however, pathologically increased mechanical forces, as observed in hypertension, lead to adaptive changes in vascular cells that may cause endothelial dysfunction and vascular disease.[7]

During the past years, several studies have demonstrated an increased production of reactive oxygen species (ROS) by endothelial cells exposed to pathological cyclic stretch.[8–10] An increased production of ROS perturbs the physiological redox balance, inducing oxidative stress resulting in inactivation of nitric oxide (NO).[11] This in turn leads to endothelial dysfunction, a key step in the pathophysiology of many cardiovascular diseases.[12]

p66^{Shc} belongs to the mammalian Shc adaptor protein family,[13,14] participates in ROS production,[15–19] and is involved in translation of oxidative stimuli into apoptosis.[20,21] Genetic deletion of p66^{Shc} in mice was shown to improve endothelial function in different disease models.[15,16,18] In particular, p66^{Shc} mediates vascular dysfunction observed in angiotensin II–treated, hypertensive mice.[22] Nevertheless, the role of p66^{Shc} in mediating the deleterious effects of pathological cyclic stretch on the vasculature is not known.

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The present study was designed to characterize the role of p66^{Shc} in cyclic stretch–induced endothelial damage. In particular, we assessed whether p66^{Shc} mediates the increased superoxide anion (O_2^-) production and the decreased NO bioavailability observed in endothelial cells exposed to pathological cyclic stretch. Finally, to assess the relevance of our findings in vivo, we explored whether p66^{Shc} activation is increased in isolated aortic endothelial cells of spontaneously hypertensive rats (SHR).

Materials and Methods

Detailed Materials and Methods are available in the online-only Data Supplement.

Cell Culture Experiments

For all in vitro experiments, primary human aortic endothelial cells (HAECs; Lonza) from passages 6 to 9 were used. Detailed Methods are provided in the online-only Data Supplement.

siRNA Transfection

Cells were transfected with predesigned small interfering RNA (siRNA) against p66^{Shc} using N-TER Nanoparticle siRNA Transfection System (Sigma-Aldrich) or by using Lipofectamine RNAiMAX Reagent (Invitrogen). Detailed Methods are provided in the online-only Data Supplement.

Application of Cyclic Stretch

Cyclic stretch to adhering cells on BioFlex culture plates (Flexcell International) was applied using the Flexcell FX-4000T system (Flexcell International). Detailed Methods are provided in the online-only Data Supplement.

Immunoblotting

Protein expression and activation were determined by immunoblot analysis. Cells were lysed in lysis buffer, and equal amounts of protein were separated by SDS-PAGE. Detailed Methods are provided in the online-only Data Supplement.

Measurement of O_2^- Production and NO Bioavailability

O_2^- production and NO bioavailability in intact cells or aortic tissue were determined using electron spin resonance spectroscopy. Detailed Methods are provided in the online-only Data Supplement.

NADPH Oxidase Activity Measurement

Determination of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation was performed using a commercially available kit (Abcam) and performed according to the manufacturer's recommendations.

Animal Experiments

Sixteen-week-old male SHR and male Wistar-Kyoto (WKY) rats were purchased from Janvier Laboratories. Study design and experimental protocols were approved by the Cantonal Veterinary Office of the Canton of Zurich. Detailed Methods are provided in the online-only Data Supplement.

Statistical Analysis

Data are presented as mean±SEM. Statistical analysis for comparison of 2 groups was performed using 2-tailed unpaired Student *t* test or Mann–Whitney test when appropriate. For comparison of >2 unmatched groups, 1-way ANOVA followed by the Bonferroni post hoc test or Kruskal–Wallis test followed by the Dunn post hoc test was performed when appropriate. A value of $P<0.05$ denoted a significant difference. Statistical analysis was performed using GraphPad Prism software 5.01.

Results

Cyclic Stretch Enhances p66^{Shc} Activation in a Stretch- and Time-Dependent Manner

Exposure of HAECs to pathological cyclic stretch (8% and 15% stretch) for 6 hours resulted in a stretch-dependent increase in p66^{Shc} phosphorylation at Ser36 compared with static condition, whereas no change in p66^{Shc} activation was observed under physiological stretch (2%; Figure 1A). In addition, the extent of p66^{Shc} activation was time-dependent as observed after exposure of HAECs to 1, 3, or 6 hours of pathological cyclic stretch (Figure 1B). All conditions did not change p66^{Shc} protein expression (Figure 1A and 1B). To exclude potential cytotoxic effects of pathological cyclic stretch, a colorimetric assay for detection of lactate dehydrogenase release was performed. No cytotoxicity was observed when cells were exposed to pathological cyclic stretch compared with static condition (data not shown).

MAPK JNK1/2 Mediates Pathological Stretch–Increased p66^{Shc} Activation

Immunoblot analysis revealed an enhanced activation of the mitogen-activated protein kinases (MAPKs) c-Jun N-terminal kinase (JNK) 1/2 (Figure 2A), p38, and extracellular signal–regulated kinase (ERK) 1/2 (data not shown) after exposure of HAECs to pathological stretch. Differently, no change in the phosphorylation (at T641) of protein kinase C βII was observed (data not shown). To characterize intracellular signaling pathways mediating pathological stretch–increased p66^{Shc} activation, MAPK JNK1/2, p38, or ERK1/2 was inhibited by incubating HAECs with SP600125 (1 μmol/L), SB203580 (1 μmol/L), or PD98059 (1 μmol/L), respectively,

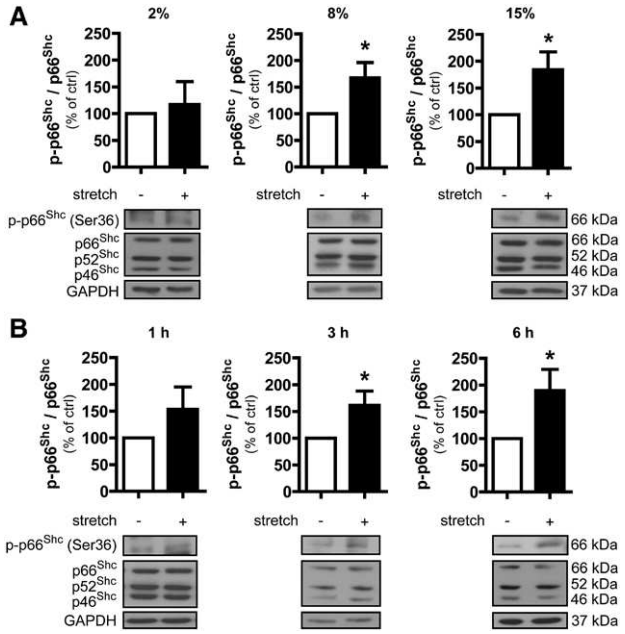

Figure 1. Effect of cyclic stretch on p66^{Shc} activation. **A**, Pathological cyclic stretch (8% and 15%; n=6), but not physiological stretch (2%; n=6), leads to an increased p66^{Shc} phosphorylation at Ser36. **B**, Human aortic endothelial cells exposed to 15% of cyclic stretch show a time-dependent increase in p66^{Shc} activation (n=6–7). *$P<0.05$ for stretch vs control. Ctrl indicates control (static condition).

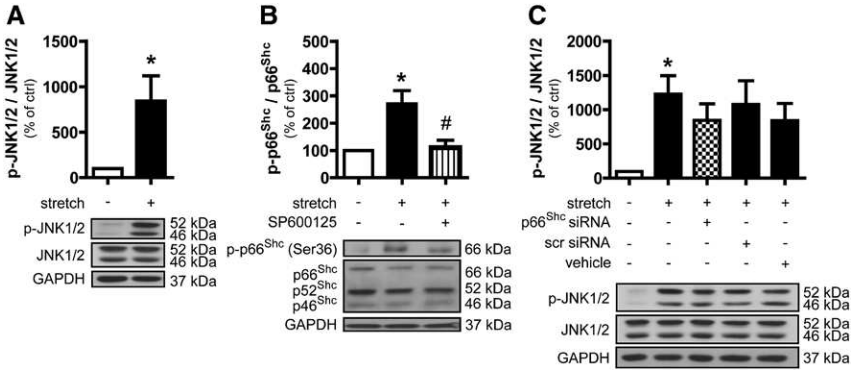

Figure 2. Mitogen-activated protein kinase c-Jun N-terminal kinase 1/2 (JNK1/2) mediates pathological stretch–increased p66Shc activation. **A**, Pathological stretch leads to an increase in the activation of JNK1/2 (n=6). **B**, Pretreatment of human aortic endothelial cells with the specific JNK1/2 inhibitor SP600125 (1 μmol/L) abolishes pathological stretch–increased p66Shc activation (n=6). **C**, Stretch-increased JNK1/2 activation is not altered after silencing of p66Shc (n=6). *P<0.05 for stretch vs control; #P<0.05 for stretch with SP600125 vs stretch alone. Ctrl indicates control (static condition); p-JNK1/2, phosphorylated JNK1/2; and scr siRNA, scrambled small interfering RNA.

before exposure to pathological stretch. Inhibition of JNK1/2 prevented stretch-increased p66Shc activation (Figure 2B). In contrast, this was not observed when p38 or ERK1/2 was inhibited (data not shown). To assess whether p66Shc activation in response to pathological cyclic stretch occurs upstream or downstream of JNK1/2, the effect of p66Shc silencing on pathological stretch–increased JNK1/2 activation was assessed. Preincubation of HAECs with siRNA-specific targeting p66Shc did not affect JNK1/2 activation and JNK1/2 protein levels under pathological stretch condition (Figure 2C), indicating that p66Shc acts as a downstream target of JNK1/2 when cells are exposed to pathological cyclic stretch.

JNK1/2, p66Shc, and NADPH Oxidase Mediate Pathological Stretch–Increased O_2^- Production and Stretch-Reduced NO Bioavailability

To assess the effect of p66Shc silencing or JNK1/2 inhibition on stretch-increased O_2^- production and stretch-reduced NO bioavailability, HAECs were transfected with siRNA-specific targeting p66Shc or pretreated with SP600125 (1 μmol/L). Silencing of p66Shc or JNK1/2 inhibition reduced stretch-increased O_2^- generation (Figure 3A) and resulted in an increased NO bioavailability under pathological stretch condition (Figure 3C). Furthermore, p66Shc silencing reduced stretch-increased NADPH oxidase activity (Figure 3B). Previously, a role for p66Shc in inhibiting endothelial NO synthase (eNOS)–dependent NO production was demonstrated.[23] Here, we investigated the potential effect of p66Shc silencing on eNOS under pathological stretch condition. Immunoblotting did not reveal any influence of p66Shc silencing under stretch condition on both Ser1177 (Figure 3D) and Thr495 (Figure 3E) phosphorylation sites of eNOS or on eNOS protein expression (Figure 3D and 3E).

Functional Integrin $\alpha_5\beta_1$ Inhibition Diminishes Both p66Shc and JNK1/2 Activation Increased by Pathological Stretch

Integrins are known to translate extracellular mechanical stimuli into intracellular adaptations.[24] To address the role of integrin $\alpha_5\beta_1$ in pathological stretch–increased p66Shc activation, its function was blocked using an anti-integrin $\alpha_5\beta_1$ antibody[25] (10 μg/mL) before pathological stretch exposure. Thereby, pathological stretch–increased p66Shc activation was reduced (Figure 4A). In line with this, pathological stretch–increased JNK1/2 activation was decreased by functional integrin $\alpha_5\beta_1$ inhibition (Figure 4B).

p66Shc Activation Is Elevated in the Endothelium of Hypertensive Rats

To test the physiological relevance of the data obtained in vitro, regulation of p66Shc was studied in vivo by assessing its activation in aortic endothelial cells of SHR compared with normotensive WKY rats. Blood pressure monitoring showed elevated systolic blood pressures in SHR compared with WKY rats, whereas no difference in heart rate was observed (data not show). Immunoblot analysis revealed an increase in p66Shc phosphorylation at Ser36 in aortic endothelial cells of SHR compared with WKY rats, without showing altered p66Shc protein expression (Figure 5A). In addition, in aortic homogenates of SHR, we observed an increased O_2^- generation (Figure 5B) as well as a reduction in NO bioavailability (Figure 5C) compared with WKY.

Discussion

This study demonstrates for the first time that pathological cyclic stretch causes an increased endothelial O_2^- production and a reduced NO bioavailability through the activation of the adaptor protein p66Shc downstream of integrin $\alpha_5\beta_1$ and JNK1/2 and upstream of the NADPH oxidase. Next, we confirm that p66Shc activation is increased also in isolated aortic endothelial cells of SHR compared with WKY rats.

Vascular cells are constantly exposed to mechanical forces, such as shear stress and cyclic stretch.[5] The location of integrins between the extracellular matrix and intracellular cytoskeleton elements assigns them an important function as mechanotransducers.[26,27] Integrins were shown to be crucial in translating pathologically increased mechanical stimuli into morphological and intracellular adaptations.[24,28] In this study, we focused on investigating the role of $\alpha_5\beta_1$ integrin, which is known to be abundantly expressed in endothelial cells[29] and to adhere to fibronectin.[30] Functional blockade of integrin $\alpha_5\beta_1$ abolished pathological stretch–increased p66Shc phosphorylation and JNK1/2 activation, demonstrating that integrin $\alpha_5\beta_1$ acts as a key mechanotransducer for the pathological endothelial adaptations observed in the present study (Figure 6).

Pathways mediating extracellular signals to crucial endothelial proteins may involve protein kinase C[31] and the MAPKs JNK, p38, and ERK.[32] A role for protein kinase C[33] and

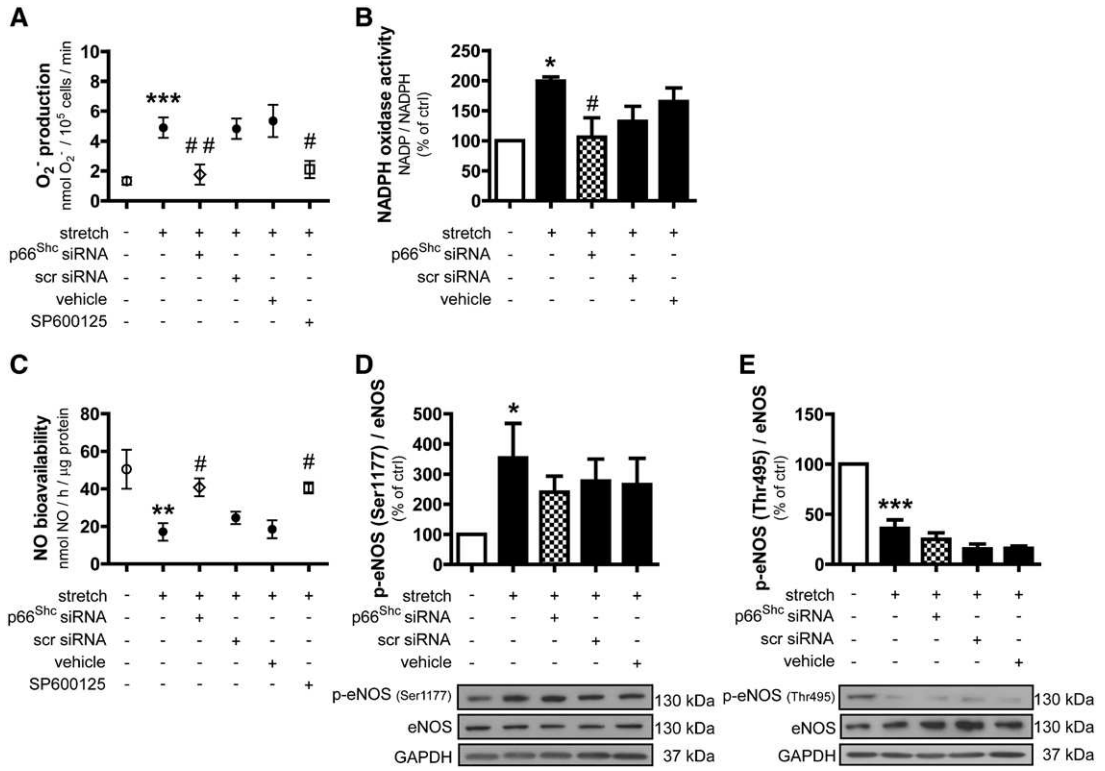

Figure 3. p66^{Shc} silencing and c-Jun N-terminal kinase 1/2 (JNK1/2) inhibition abolish pathological cyclic stretch–increased O_2^- production and increases pathological stretch–reduced nitric oxide (NO) bioavailability. **A**, Pretreatment of human aortic endothelial cells with specific small interfering RNA (siRNA) against p66^{Shc} or with the pharmacological JNK1/2 inhibitor SP600125 (1 µmol/L) reduces pathological stretch–increased O_2^- production (n=6–12). **B**, Pathological stretch–increased nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity is significantly reduced after silencing of p66^{Shc} (n=4). **C**, Both p66^{Shc} silencing and JNK1/2 inhibition increase NO bioavailability under pathological stretch condition (n=6). p66^{Shc} silencing does not alter endothelial NO synthase (eNOS) phosphorylation at Ser1177 (n=7; **D**) and Thr495 (n=4; **E**) or eNOS protein expression under stretch condition. *P<0.05, **P<0.01, ***P<0.001 for stretch vs control; #P<0.05, ##P<0.01 for stretch with p66^{Shc} silencing or SP600125 vs stretch alone. Ctrl indicates control (static condition); p-eNOS, phosphorylated endothelial NO synthase; and scr siRNA, scrambled small interfering RNA.

MAPKs[34] in endothelial cell adaptations induced by pathological cyclic stretch has been reported previously. Furthermore, JNK is a known activator of p66^{Shc} after stimulation of endothelial cells with oxidized low-density lipoprotein.[35] Unlike protein kinase C βII, phosphorylation of JNK1/2, p38, and ERK1/2 was enhanced after exposure to pathological stretch. However, only inhibition of JNK1/2, and not inhibition of p38 or ERK1/2, blunted p66^{Shc} activation and O_2^- production and restored NO bioavailability after pathological stretch, thus indicating a pivotal role for JNK1/2 in these settings.

An increased level of endothelial ROS after pathological stretch exposure, as observed in our study, is in line with previous investigations.[8,9] A balance between ROS production and degradation is essential to maintain

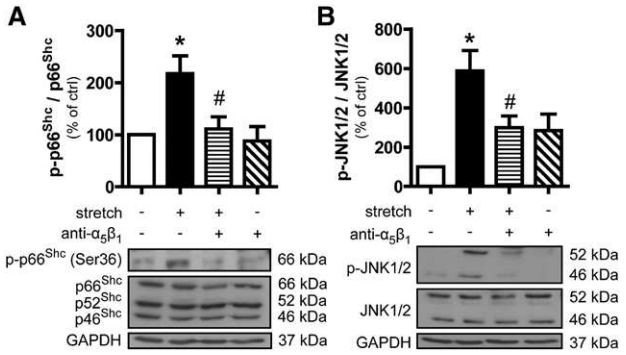

Figure 4. Role of integrin $\alpha_5\beta_1$ on pathological stretch–increased p66^{Shc} and c-Jun N-terminal kinase 1/2 (JNK1/2) activation. **A** and **B**, Functional integrin $\alpha_5\beta_1$ blocking abolishes p66^{Shc} (n=5–6) and JNK1/2 (n=7–8) activation induced by pathological stretch. *P<0.05 for stretch vs control; #P<0.05 for stretch with anti$\alpha_5\beta_1$ vs stretch alone. Ctrl indicates control (static condition); and p-JNK1/2, phosphorylated JNK1/2.

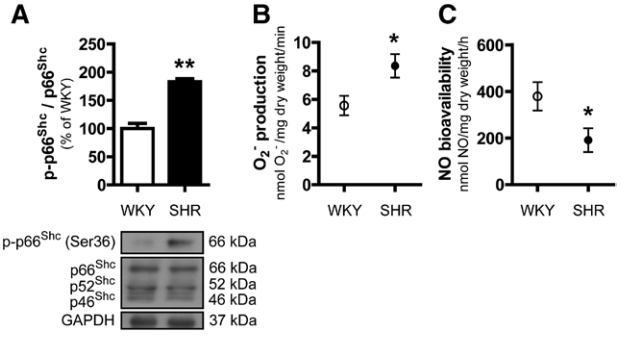

Figure 5. p66^{Shc} activation in aortic endothelial cells of Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR). **A**, Immunoblot analysis reveals an increase in p66^{Shc} activation (n=4–6) in aortic endothelial cells of hypertensive rats compared with normotensive ones. **B** and **C**, SHR show elevated levels of O_2^- (n=6) and reduced nitric oxide (NO) bioavailability (n=6) in aortic homogenates compared with WKY rats. *P<0.05, **P<0.01 for SHR vs WKY.

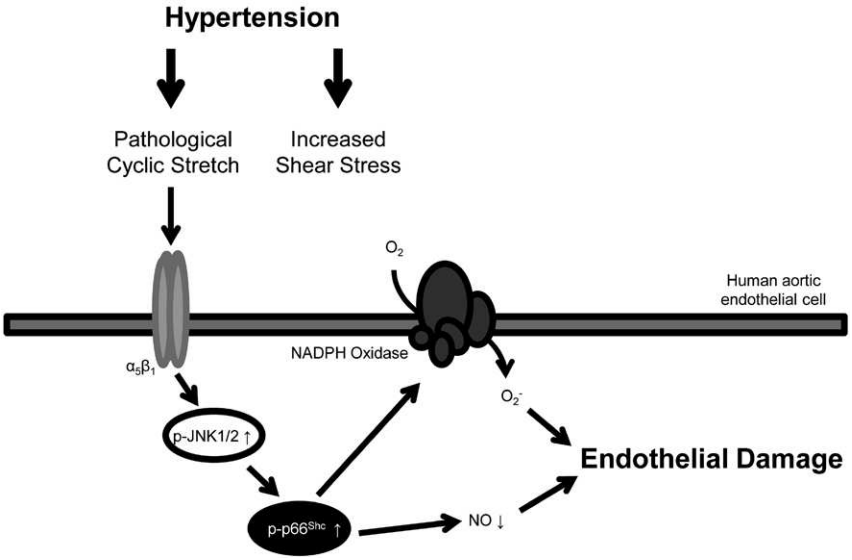

Figure 6. Role of p66Shc protein in pathological cyclic stretch–induced endothelial damage. Exposure of primary human aortic endothelial cells to pathological stretch, as it occurs in hypertension, elevates c-Jun N-terminal kinase 1/2 (JNK1/2) activation, p66Shc phosphorylation at Ser36, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity that in turn augments O_2^- production and reduces nitric oxide (NO) bioavailability, inducing endothelial damage. Nearby, integrin $\alpha_5\beta_1$ acts as a mechanotransducer for pathological stretch–induced endothelial adaptations. p-JNK1/2 indicates phosphorylated JNK1/2.

physiological cellular processes and function,[21] and the perturbation of this redox balance, by an increased ROS production and a decreased antioxidant capacity, induces oxidative stress resulting in endothelial dysfunction,[21] a common denominator to many cardiovascular diseases, such as hypertension.[36,37] Previously, NADPH oxidase has been demonstrated to be a major source for pathological stretch–increased O_2^- generation.[8] Furthermore, NADPH oxidase was demonstrated to be a downstream target of p66Shc,[35] thus NADPH oxidase may represent the source of stretch-increased O_2^- production downstream of p66Shc. In the present study, we showed that silencing of p66Shc prevents stretch-increased NADPH oxidase activity, suggesting that pathological stretch leads to an increase in p66Shc activation, which in turn activates NADPH oxidase and as a consequence increases O_2^- generation.

NO is a key mediator of endothelial function and thereby of vascular health.[38] A large body of evidence indicates that hypertensive animals[39–43] and patients with hypertension[36,37] are characterized by endothelial dysfunction. NO is rapidly inactivated by O_2^- to form peroxynitrite, exerting nitrosative stress to the cell.[11] In line with a previous report,[44] NO bioavailability was decreased by pathological cyclic stretch that was, in the present study, prevented by p66Shc silencing, independent of eNOS activation. This finding, together with the above described O_2^- data, supports the idea that the observed decrease in NO levels after pathological stretch is the result of the scavenging action of O_2^- on NO, rather than the result of a changed NO production, and in turn, the increased NO bioavailability on p66Shc silencing is the result of a decreased O_2^- production.

Finally, we studied the regulation of p66Shc in aortic endothelial cells of SHR, whereby cyclic stretch is known to occur, to test the in vivo relevance of our in vitro data. In these settings, we were able to confirm our in vitro findings by demonstrating increased p66Shc activation in aortic endothelial cells of SHR compared with WKY rats. Furthermore, as observed in HAECs, we could confirm in aortic homogenates of SHR an increase in O_2^- production and a decrease in NO bioavailability compared with WKY rats underscoring that the pathways elucidated in vitro are also relevant in vivo. These findings are in line with a previous study showing increased activation of p66Shc in the rat coarctation model of hypertension.[45]

Study Limitations

In the present study, we demonstrate an increased activation of p66Shc in aortic endothelial cells of hypertensive rats, where pathological cyclic stretch occurs. However, there is no clear evidence that this effect is mediated solely by increased cyclic stretch because other factors involved in hypertension, such as shear stress, occur in parallel in the vessels of SHR.

Perspectives

Hypertension is a major risk factor for myocardial infarction and stroke.[1] One in 3 adults is diagnosed with hypertension,[1] thus a clear understanding of the molecular mechanisms underlying hypertension-induced vascular disease is essential for its prevention and treatment. The present study offers new insights into the signaling pathways that may be responsible for mediating the noxious effects of pathological cyclic stretch on endothelial function[36,37] and demonstrates a crucial role of the adaptor protein p66Shc in this context.

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Disclosures

None.

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Novelty and Significance

What Is New?

- The adaptor protein p66Shc, on activation by c-Jun N-terminal kinase, mediates pathological cyclic stretch–increased O_2^- production and pathological cyclic stretch–decreased NO bioavailability.

What Is Relevant?

- Patients with hypertension display endothelial dysfunction largely because of an increased reactive oxygen species production at the expense of a higher nitric oxide scavenging by O_2^-. Pathological cyclic stretch is partially responsible for this, and here we present a novel mediator that may be implicated in this phenomenon.

Summary

This study provides mechanistic insights into the signaling pathways activated by pathological cyclic stretch. In particular, we demonstrate a crucial role of p66Shc in mediating pathological cyclic stretch–increased superoxide anion production and pathological cyclic stretch–reduced nitric oxide bioavailability. This novel molecular pathway may be relevant for endothelial dysfunction and vascular disease in hypertension.

ONLINE SUPPLEMENT

The Adaptor Protein p66^{Shc} Mediates Hypertension Associated, Cyclic Stretch-Dependent, Endothelial Damage

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Detailed Material and Methods

Cell Culture Experiments

Primary human aortic endothelial cells (HAECs) (Lonza) were cultured in EBM-2 medium supplied with EGM-2 bullet kit (Lonza). For all experiments, adhering cells were grown to confluence on fibronectin-coated BioFlex® culture plates (Flexcell International) and rendered quiescent in EBM medium (Lonza) with 0.5% FBS. For all experiments, cells from passages 6 to 9 were used.

To block the MAP kinases p38, extracelluar signal-regulated kinase p44/42 (ERK1/2), or c-Jun N-terminal kinase (JNK1/2), cells were treated with SB203580 (Sigma), PD98059 (Cell Signaling), or SP600125 (Calbiochem) respectively, before pathological stretch exposure. For functional integrin alpha5beta1 ($\alpha_5\beta_1$) blocking, cells were pre-incubated with an anti-integrin $\alpha_5\beta_1$ antibody (Millipore), before pathological stretch exposure.

Small Interfering RNA (siRNA) Transfection

Cells were transfected with predesigned siRNA targeting $p66^{Shc}$ (sense: 5′-AUGAGUCUCUGUCAUCGCU[dT][dT]-3′; antisense: 5'-AGCGAUGACAGAGACUCAU[dT][dT]-3') (Sigma-Aldrich) using N-TER Nanoparticle siRNA Transfection System (Sigma-Aldrich) at final concentration of 10 nmol/L, or by using Lipofectamine®RNAiMAX Reagent (Invitrogen) at final concentration of 25 nmol/L (for NADPH oxidase activity measurements), according to the manufacturer's protocol. siRNA incubation was performed using serum-free and antibiotics-free EBM-2 medium (Lonza) for 4 h, followed by 28 h of incubation in normal growth medium. As negative controls, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) siRNA (sense: 5′-GGUUUACAUGUUCCAAUAU[dT][dT]-3′; antisense: 5'-AUAUUGGAACAUGUAAACC[dT][dT]-3') (Sigma-Aldrich), scrambled siRNA (sense: 5'-GAUCAUACGUGCGAUCAGA[dT][dT]-3'; antisense: 5'-UCUGAUCGCACGUAUGAUC[dT][dT]-3') (Sigma-Aldrich), and vehicle alone were used.

Application of Cyclic Stretch

Adhering cells on the flexible membrane of BioFlex® culture plates (Flexcell International) were exposed to different cyclic stretch (0.5 Hz) regimens (2%, 8%, or 15%) with different interval times (1 h, 3 h, 6 h) using the Flexcell® FX-4000T™ system (Flexcell International). For all ROS, NO, and NADPH oxidase activity measurements as well as mechanistic experiments, a pathological stretch regimen of 15% and 6 h was used. To assess cytotoxicity, a colorimetric assay (Roche) for detection of lactate dehydrogenase (LDH) release was used according to the manufacturer's recommendations.

Immunoblotting

Protein expression and activation was determined by immunoblot analysis. Cells were lysed in 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L NaF, 1 mmol/L DTT, 10 µg/µl aprotinin, 10 µg/µl leupeptin, 0.1 mmol/L Na_3VO_4, 1 mmol/L PMSF, and 0.5% NP-40. For analyzing protein kinase C beta II (PKCβ II) phosphorylation, cells were lysed in 20 mmol/L MOPS, 50 mmol/L β-glycerol phosphate, 50 mmol/L NaF, 10 µg/µL aprotinin, 10 µg/µL leupeptin, 1 mmol/L Na_3VO_4, 5 mmol/L EGTA, 2 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L PMSF, 1 mmol/L benzamide, and 1% NP-40. Protein concentration was measured according to the manufacturer's recommendations (Bio-Rad). Separation of proteins was done by SDS-PAGE, followed by the transfer to a polyvinylidene fluoride membrane (Millipore) by semidry transfer. Antibodies against phosphorylated p38, ERK1/2, and JNK1/2 (all from Cell Signaling) were used at 1:1000, 1:5000, and 1:1000 dilution, respectively. Antibodies against p38, ERK1/2, and JNK1/2 protein expression (all from Cell Signaling) were used at 1:2000, 1:5000, and 1:1000 dilution, respectively. Anti-Shc/p66 (pSer36) antibody (Calbiochem) was used at 1:100 dilution. Anti-pT641PKCβ II (Invitrogen) and anti-Shc (Cell Signaling, or BD Transduction Laboratories™) antibodies were used at 1:1000 dilution. Anti-PKCβ II antibody (Santa Cruz) was used at 1:1000 dilution. Antibodies against p-eNOS (Thr495), p-eNOS (Ser1177), and eNOS (all from BD Transduction Laboratories™) were used at 1:5000, 1:2000, and 1:2000 dilution. Immunoblots were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:20000 dilution; Millipore) expression. Anti-rabbit and anti-mouse secondary antibodies were purchased from SouthernBiotech.

Measurement of O_2^- Production and NO Bioavailability

O_2^- production was determined using electron spin resonance (ESR) spectroscopy, as previously described [1]. Intact cells were suspended in Krebs-HEPES solution (Noxygen) containing 200 µmol/L 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine hydrochloride (Noxygen), 5 µmol/L sodium diethyldithiocarbamate trihydrate (Noxygen), and 25 µmol/L deferoxamine methanesulfonate (Noxygen). On an ESR spectrometer (Bruker), O_2^- production was measured using following instrumental settings: center field, 3472.0 G; sweep width, 10.00 G; microwave frequency, 9.76 GHz; microwave power, 19.91 mW; modulation frequency, 86.00 kHz; modulation amplitude, 2.60 G; and number of scans, 10. The temperature was set constant at 37° Celsius (temperature-controlled system). Aortic tissue of SHR and WKY rats was incubated with Krebs-HEPES solution (Noxygen) containing 500 µmol/L 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine hydrochloride (Noxygen), 5 µmol/L sodium diethyldithiocarbamate trihydrate (Noxygen), and 25 µmol/L deferoxamine methanesulfonate (Noxygen) for 1 h at 37ºC. Samples frozen in liquid nitrogen were measured on an ESR spectrometer (Bruker) by using following instrumental settings: sweep width, 50.000 G; microwave frequency, 9.78 GHz; microwave power, 19.91 mW; modulation frequency, 86.00 kHz; modulation amplitude, 2.60 G; and number of scans, 10.

NO bioavailability was determined using ESR spectroscopy, as previously described [1, 2]. $Fe(DETC)_2$ was formed by equal volumes of diethyldithiocarbonic acid sodium salt (7.2 mg/10 ml 0.9% NaCl) (Noxygen) and ferrous sulphate heptahydrate (4.5 mg/10 ml 0.9% NaCl) (Noxygen). HAECs, or aortic tissue were incubated with Krebs-HEPES – $Fe(DETC)_2$ mixture for 1 h at 37ºC and then collected in Krebs-HEPES solution (Noxygen), followed by snap freezing in liquid nitrogen. Frozen samples were measured on an ESR spectrometer (Bruker) using following instrumental

settings: center field, 3455.00 G; sweep width, 80.00 G microwave frequency, 9.78 GHz; microwave power, 39.72 mW; modulation frequency, 86.00 kHz; modulation amplitude, 10.34 G; and number of scans, 10.

Animal Experiments

Male spontaneously hypertensive rats (SHR) and male Wistar-Kyoto rats (WKY), all of which 16 weeks old, were purchased from Janvier Labs. Animals had *ad libitum* access to food (normal chow diet) and water, and were housed under a 12 h light/dark cycle. Following habituation training of the animals, systolic blood pressure (SBP) and heart rate (HR) measurements were performed by tail-cuff method (model LE 5002; Letica). Following anaesthesia by pentobarbital (50 mg/kg i.p.), animals were sacrificed by blood exsanguinations. Aortic tissue was then collected for measurement of O_2^- production and NO bioavailability, and rat aortic endothelial cells were isolated from the aorta for immunoblotting. Study design and experimental protocols were approved by the Cantonal Veterinary Office of the Canton of Zurich.

Isolation of rat aortic endothelial cells

Isolated aorta from SHR and WKY rats were quickly washed in Hanks's Balanced Salt Solution (HBSS) (Invitrogen) containing 10 mmol/L HEPES and 0.1% BSA. Aorta were opened by a longitudinal cut and incubated on a culture dish (TPP®) treated with 0.4 % collagenase-dispase solution (Roche) for 35 min at 37°C. Resulting endothelial cell fraction was centrifuged for 5 min at 233 g and 4 °C. Pellet was resuspended in growth medium (DMEM (Life Technologies™) containing 20% FCS, 0.1 mg/ml heparin, 25 mmol/L HEPES, 1% non-essential amino acid (Sigma), 1% penicillin-streptomycin (PAA Laboratories GmbH), supplemented with 0.1 mg/ml of endothelium cell growth supplement (Sigma-Aldrich)), and plated on a culture dish (TPP®), pre-treated with 0.1% of gelatine of bovine skin origin (Sigma-Aldrich). Cells were cultured to confluence in growth medium and then lysed for immunoblotting.

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Supplementary Figures

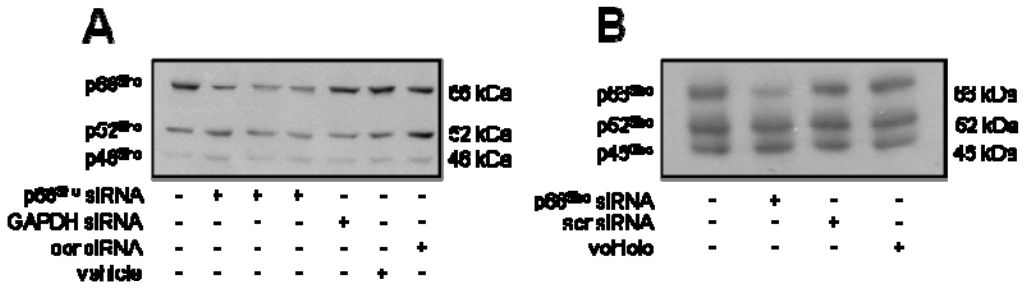

Figure S1. Representative immunoblot of p66^{Shc} silencing. Protein levels of p66^{Shc}, but not of p52^{Shc} and p46^{Shc}, are selectively reduced after transfecting HAECs with specific siRNA against p66^{Shc}. A, N-TER Nanoparticle siRNA Transfection System (Sigma-Aldrich). B, Lipofectamine®RNAiMAX Reagent (Invitrogen). scr siRNA indicates scrambled siRNA.

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